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(71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US).

(72) Inventors: LEDER, Philip ; 25 Aston Road, Chestnut Hill, MA 02167 (US). TEPPER, Robert ; Two Hawthorne Place, #5R, Boston, MA 02114 (US). LEVINSON, Douglas, A. ; 111 Perkins Street, Apt. 22-1, Jamaica Plain, MA 02130 (US).

(74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

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(54) Title: TRANSGENIC MOUSE OVEREXPRESSING IL-4 AND METHOD OF USE

(57) Abstract

A transgenic non-human vertebrate animal having cells containing a transgene encoding IL-4, which transgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage; and methods of controlling the level of expression of a transgene in a transgenic animal.

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TRANSGENIC MOUSE OVEREXPRESSING IL-4 AND METHOD OF USE

Background of the Invention

The field of the invention is transgenic animals.

The cytokines are highly pleiotropic effectors of immunity and inflammation which play a major role in the growth and differentiation of lymphoid and hematopoietic cells responding to a foreign antigenic challenge. In vitro studies have indicated that one cytokine in particular, termed interleukin-4 or IL-4, exerts a broad range of proliferative and differentiation-inducing activities in both mature and immature T cell subsets, B cells, and hematopoietic precursors (reviewed by Paul, FASEB 1:456-461, 1987).

Summary of the Invention

In general, the invention features, in one aspect, a transgenic non-human vertebrate animal (preferably a mammal such as a rodent, e.g., a mouse) having cells (e.g., somatic cells and germline cells) coutaining a transgene encoding IL-4, which transgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage. A "transgene" is defined as a piece of DNA which is inserted by artifice (i.e., by a means other than sexual propagation) into a cell, and becomes part of the genome of the animal which develops from that cell. Such a trensgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic animal, or may represent a gene homologous to a natural gene of the transgenic animal, but which is inserted into the animal's genome at a location which differs from that of the natural homolog. A "trensgenic animal" is an animal having cells that contain a transgene, which

transgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage. By "embryonic stage" is meant any point from the moment of conception (e.g., as where the sperm or egg bears the transgene) throughout all of the stages of enbryonic development of the fetus, and preferably refers to a stage within the first eight days following conception.

In preferred embodiments, the transgene contains a heterologous promoter region: for example, a mammalian immunoglobulin promoter region (which may include both a mammalian immunoglobulin enhancer and a mammalian immunoglobulin promoter), or a promoter region which includes the SV40 eanly region promoter; the promoter of a mammalian actin gene, CD-2 gene, c-fos gene, Thy-1 gene, elastase gene, or metallothionein gene; or the long- terminal repeat of the mouse mammary tumor virus (MMTV-LTR). Preferably, the heterologous promoter region does not contain a naturally-occurring IL-4 promoter; the IL-4 is a mammalian IL-4 (e.g., that of a mouse or of a human); the transgene is expressed predominantly in lymphoid tissues of the transgenic animals [i.e., transgene mRNA is more abandant in lymphoid tissues (e.g., spleen, thymus, and lymph nodes) than in other types of tissues of the transgenic animals]; the transgenic animal exhibits a heightened allergic response compared to wild-type animals of the same species; and the transgenic animal is pridisposed to develop an inflammatory lesion of the eyelid. Those transgenic animals which exhibit a heightened allergic response may be used in a method for testing an anti-allergy treatment, which method involves exposing the animal to the anti-allergy treatment and determining the effect of the treatment on the allergic response of the animal. A "promotor" is a segment of DNA 5' to the transcription start site of a gene, to which RNA polymerase binds

before initiating transcription of the gene. By "promoter region" is meant the entire part of the transgene which is 5' to the transcription start site of that transgene. A "heterologous promoter region" is a promoter region that is not identical to the corresponding naturally-occurring promoter region for the given gene (e.g., a promotor region which does not include the naturally-occurring promotor).

The invention also features an "operator^{+}/ represser^{-}" transgenic non-human vertebrate animal (preferably a mammal such as a rodent, e.g., a mouse) having cells containing a transgene having an untranslated region (consisting of all parts of the transgene which are not translated into protein, including the promoter region, the 5' untranslated region [between the transcription start site and the initiation codon], and all introns, which includes a heterologous DNA segment of 6 or more (e.g., up to 100) base pairs, which heterologous DNA segment consists of a prokaryotic or eukaryotic operator, a eukaryotic transcription factor binding site, a palindromic sequence, or a sequence having dyad symmetry, which trensgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage. This heterologous DNA segment functions to reduce, or "attenuate," the level of transcription of the transgene, a function which is useful, for example, where a mouse with unattenuated expression of the transgene would be incapable of sexual reproduction (as where such unattenuated expression is fatal to the transgenic mouse, or otherwise prevents it from reaching sexual maturity).

In preferred embodiments, the heterologous DNA segment is present as one to ten copies in the untranslated region (more preferably, one to three copies), and is selected from the following group: the operator of the E. coli lac operon, the E. coli tet

operon, the E. coli met operon, or the E. coli gal operon; the phage lambda operator; the phage 434 operater; the phage 21 operator; the phage 22 operator; the yeast STE6 operator; the dyad symmetry element of the human c-fos promoter; the AP-1 transcription factor binding site; the estrogen receptor binding site; a palindromic sequence of 8-50 base pairs, such as a palindromic fragment of one of the aforementioned operators; or a sequence of 8-50 base pairs having dyad symmetry, such as a fragment of one of the aforementioned operators, having dyad symmetry. Most preferably, the heterologous DMA segment is located in the promoter region, and includes the operator of the E. coli lac operon or, alternatively, the following palindromic sequence:

ATTGTGAGCGGTGAGAAT
TAACACTCGCGAGTGTAA

In another aspect, the invention features a transgenic non-human vertebrate animal (preferably a mammal such as a rodent; e.g., a mouse) having cells (e.g., somatic cells and germline cells) containing a transgene encoding a heterologous repressor protein (e.g., the E. coli lac repressor protein), which transgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage. A repressor protein is a protein which is capable of binding to a specific DNA sequence within a given gene, thereby reducing (i.e., "repressing") the level of transcription of that gene. A "heterologous repressor protein" is a repressor protein which meets one or both of the following criteria: (1) it is a repressor protein other than one which is synthesized by wild-type animals of the same species as the transgenic animal, or (2) the gene encoding it is inserted into a position in the genome of the transgenic animal other than the position of the

corresponding natural gene of wild-type animals of the same species. Animals transgenic only for such a heterologous repressor protein gene are referred to as "operator$^-$/repressor$^+$" transgenic animals. Preferably, the cells also contain a second transgene having an operator sequence to which the heterologous repressor protein is capable of binding, in which case the animal is herein termed "operator$^+$/repressor$^+$". These operator$^+$/repressor$^+$ animals are preferably made by the following method: (1) a transgenic non-human vertebrate recipient animal is provided which has cells containing a transgene encoding a repressor protein (e.g., the E. coli lac repressor protein), which transgene was introduced into said recipient animal, or an ancestor of said recipient animal, at an embryonic stage; and (2) an additional transgene (preferably IL-4 or human growth hormone) is introduced at an embryonic stage, into a descendant of the recipient animal, the sequence of which additional transgene includes an operater sequence (e.g., the operator of the E. coli lac operon, or the following palindromic sequence:

ATTGTGAGCGCTCACAAT
TAACAGACGCGAGTGTTA

to which the repressor protein is capable of binding, thereby reducing the level of expression of the additional transgene in the double-transgenic animal. Such operator$^+$/repressor$^+$ transgenic animals may be made either by sexually crossing an operator$^+$/repressor$^-$ animal with an appropriate operator$^-$/repressor$^+$ animal, or by injecting fertilized oocytes of operator$^-$/ repressor$^+$ animals with DNA encoding a gene bearing the appropriate operator.

In preferred embodiments, the binding of the repressor protein, and thus the attenuation of

transcription, may be reversed in operator+/repressor+ double-transgenic animals by (a) mating the double-transgene animal to a wild-type animal, thereby producing offspring approximately one-half of which lack the repressor transgene, which half includes some which retain the other transgene; or (b) introducing into the double-transgenic animal an inactivator of the repressor protein: where the repressor protein is the E. coli lac repressor protein, the inactivator is isopropyl thio- β-D-galactoside ("IPTG"). The inactivator of the repressor can be any entity which, when it interacts with the repressor protein in vivo, causes the repressor protein to lose its ability to repress transcription.

The IL-4-transgenic animals of the invention, which overexpress IL-4 to varying degrees in lymphoid tissues, provides a means to study the effect of overexpression of IL-4 in a reproducible in vivo system. The tendency of a large percentage of these amimals to develop an inflammatory lesion of the eyelid having histologic features seen in allergic disorders makes the IL-4-overproducing animals of the invention a useful animal model for human allergic disorders. Such an animal model can be used to study the nature of the allergic response, and to test proposed means of preventing, controlling or curing allergies in humans.

These methods for producing transgenic animals having attenuated levels of expression of a foreign gene have general applicability to the field of transgenic animal generation, as they permit control of the level of expression of genes, full expression of which may be lethal to the subject animal, or which may otherwise prevent reproduction and maintenance of the animal's germ line. The

operator^{+}/repressor^{-} method of the invention "damps down" expression of the associated gene to a level dependent upon the number of such operators insented into the promoter region, permitting the creation of a series of strains displaying a gradient of levels of expression. The operator^{+}/repressor^{+} method of the invention provides a reversible "on-off" switch that largely prevents expression of the gene associated with the inserted operator until such time as the system is derepressed by the addition of an exogenous repressor-inactivating molecule, or by removal of the repressor gene by mating the double transgenic animal with a wild-type animal, resulting in segregation of the two transgenes in the offspring such that some (approximately 1/4) of the offspring carry the operator- bearing gene but <u>not</u> the repressor gene. The operator^{+}/repressor^{+} method ensures that transgenic strains bearing even potentially lethal transgenotypes can be easily maintained without selection against the lethal transgene. In addition, the use of the repressor inactivator to control the timing of expression of the foreign geme provides a means for investigating the effect of the particular gene product on, for example, behavior, learning, immunological stimulation or suppression, the etiology of various diseases such as cancer, or a particular stage of embryological development. Furthermore, an established animal strain expressing the transgenic repressor gene would be useful as a source of oocytes into which to transfer any gene bearing an appropriate operator sequence, or to cross sexually with a second animal already transgenic for a foreign gene bearing the operator sequence, in order to generate any desired operator^{+}/repressor^{+} double transgenic animal.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments, and from the claims.

Description of the Preferred Embodiments

The drawings are first described.

Drawings

Fig. 1 is a representation of the structures of DNA constructs used in the generation of IL-4 transgenic lines.

Fig. 2 is the sequence of a double-stranded 18bp lac operator analog with an internal SalI restriction site.

Fig. 3 is a photograph of an RNase protection analytic gel in which RNA from various lymphoid tissues of TG.UD and TG.UG heterozygotes, wild-type littermate controls, and IL-e-producing plasmacytoma cells ("I3L6") was probed with an IL-2 riboprobe which distinguishes endogenous IL-4 mRNA (183-nucleotide protected fragment) from transgenic IL-4 mRNA (212-nucleotide protected fragment).

Fig. 4 is a series of photographs of histologic sections of thymus from wild-type mice (A and C), transgenic TG.UD mice (B and D), and bone-marrow- reconstituted mice (E and F).

Fig. 5 is a flow cytometric analysis of the surface antigens of thymocytes from wild type (A); TG.UD heterozygote (B, C, D); and TG.UG mice (E), performed by double-color staining with antibodies against the indicated surface antigens.

Fig. 6 is a bar graph illustrating the proliferative response of spleen cells from four IL-4 transgenic lines and wild-type controls at three spleen cell concentrations.

Fig. 7 is a pair of bar graphs illustrating the mean concentrations of serum IgE (A) and serum IgG1 and IgG2a (B) for IL-4 tramsgenic lines and wild-type controls.

Fig. 8 is a series of photographs illustrating the inflammatory eye lesion observed in many IL-4 transgenic mice, both macroscopically (A, right side; a wild-type control mouse is shown for comparison in A, left side) and at 1000x magnification of a stained tissue section (B and C).

Fig. 9 is an illustration of the genes utilized in Example 3.

IL-4-Overexpressing Transgenic Mice

Three classes of IL-4-overexpressing transgenic mice were generated in accordance with the invention. One class, termed "operator$^-$/repressor$^-$," bears a murine IL-4 gene in which the natural promoter region of the IL-4 gene has been replaced with a promoter region heterologous to the IL-4 gene (i.e., having a sequence not identical to the natural promoter region of the IL-4 gene). This construct causes the recipient transgenic mice to overexpress IL-4 to such an extent that every founder animal of this class died within two weeks of birth.

The second class of transgenic animals is herein termed the "operator$^+$/repressor$^-$" class. The transgenic mice of this class which are described in Example 2 bear the same IL-4 gene with the same heterologous promoter region as the transgenic mice described in Example 1, but in addition have either one or three copies of an *E. coli lac* operator sequence inserted into the promoter region, which reduces the level of expression of the IL-4 gene in the recipient mouse.

The third class of transgenic animals, the "operator$^+$/repressor$^+$" class, is illustrated by the double-transgenic mice of Example 3, which bear the same lac operator-containing IL-4 transgene described in Example 3, in addition to a lac repressor transgene. Repressor protein expressed by the lac repressor transgene in the double-transgenic animal binds to the operator sequence(s) present in the IL-4 promoter region, thus repressing transcription of the IL-4 transgene in vivo.

Example 1: Operator$^-$/Repressor$^-$ IL-4 Transgenic Mice

To generate transgenic mice overexpressing IL-4, a 10.5 kb genomic fragment containing the IL-4 coding region and 3.5 kb of the 3' flanking region was linked to enhancer and promoter elements derived from the mouse and human immunoglobulin heavy chain locus, respectively (Ig.IL4; Fig. 1). The choice of the immunoglobulin control elements was based on previous studies demonstrating its transcciptional activity in both B and T lymphocyte lineages (Schmidt et al., Proc. Natl. Acad. Sci. USA 85:6047-6051, 1988; Fenton et al., Science 241:1089-1092, 1988; Langdon et al., Cell 47:11-18, 1986; Grosschedl et al., Cell 38:647-658, 1984) including adult and fetal thymocytes (Reth and Alt, Nature 312:418-423, 1984; Gallagher and Miller, Eur. J. Immunol. 18:183-186, 1988). Stable transfection of a plasmid (pIg.IL4) containing this construct into cultured plasmacytoma cells results in a high level of IL-4 production in vitro (Tepper et al., Cell 57:503-512, 1989).

Under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure, deposit of these pIg.IL-4-transfected cells has been

made with the American Type Culture Collection (ATCC) of Rockville, MD, USA, where the deposit was given Accession Number A.T.C.C. CRL 9911.

Applicants' assignee, the President and Fellows of Harvard College, represents that the ATCC is a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. All restrictions on the availability to the public of the material so deposited will be irrevocably removed upon the granting of a patent. The material will be available during the pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. §122. The deposited material will be maintained with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposited plasmid, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of the patent, whichever period is longer. Applicants' assignee acknowledges its duty to replace the deposit should the depository be unable to furnish a sample when requested due to the condition of the deposit.

A SalI-KpnI fragment of plasmid pIg.IL4, which fragment includes the Ig.IL4 construct, was prepared for microinjection as described by Sinn et al. (Cell 49:465- 475, 1987). Fertilized eggs were obtained from FVB/N females mated with FVB/N males the night prior to injection. Approximately 0.5-1 pl of DNA solution was injected into the male pronucleus. Following microinjection, grossly viable eggs were washed in M2 medium (Quinn et al., J. Reprod. Fert. 66:161-168, 1982) and transferred

into the oviducts of pseudopregnant Swiss-Webster mice. Animals were obtained from Taconic Farms (Germantown, NY).

In order to identify transgenic animals, DNA was extracted from tail sections according to a protocol modified from Davis et al. (Meth. Enzymol. 65:404-411, 1980), using a single phenol-chloroform extraction prior to ethanol precipitation. The presence of transgenic DNA was detected by Southern blot analysis (Southern, J. Mol. Biol. 98:503-517, 1975) of EcoR1-digested DNA using a 400bp StyI probe (containing the first IL-4 exon) radiolabeled with α-[^{32}P]dCTP using the random hexamer priming method (Feinberg and Vogelstein, Anal. Biochem. 132:6-13, 1983). Nine of 66 mice (14%) so analyzed carried the transgene. Despite their normal gross appearance at birth, all the founder animals displayed severe runting and died within the first two weeks of life. The postnatal lethal phenotype was poorly characterized due to the rapid demise of the animals; however, gross and histologic analysis of a single founder (TG.TA) demonstrated severe hypoplasia of the thymus with absence of a definable cortex and medulla and marked lymphocyte depletion in the spleen.

Example 2: Operator$^+$/Repressor$^-$ IL-4 Transgenic Mice

In an attempt to establish viable, IL-4-overexpressing transgenic lines, constructs were designed which were analogous to Ig.IL4 except that they had, in order to attenuate expression from the promoter, single or multiple copies of a lac operator (lac O) sequence from E. coli inserted into the immunoglobulin promoter. The constructs were prepared as follows: a double-stranded oligonucleotide which

encodes an 18 bp lac operator analog (Fig. 2) was synthesized (Sadler et al., Proc. Natl. Acad. Sci. USA 80:6785-6789, 1983). An internal SalI restriction site was engineered at the 3' and for the purpose of cloning additional operator sequences. The oligonucleotides were inserted into the 3' BanI site within the human IgH promoter of the Ig.IL4 fragment and cloned into the plasmid vector pBluescript (pBT; Stratagene, La Jolla, CA). In the resulting plasmid, pIg.O.IL4, the introduced operator sequence resides between the TATA box region of the promoter and the usual transcriptional start point (see Fig. 1). The triple operator construction, pIg.03.IL4, was created by cloning a tandem repeat of the operator sequence into the SalI site of pIg.O.IL4, which lies immediately 3' of the already-existing lac O sequence in the promoter (see Fig. 1). Both plasmids contain a SalI-XmaI fragment of pBT polylinker sequence immediately 5' of the first IL-4 exon. The Ig.O.IL4 and Ig.03.IL4 recombinants used for microinjection (Fig. 1) were isolated from their respective plasmids by digestion with NotI and Asp718. IL-4 expression in J558L plasmacytoma cells transiently transfected with either of these two constructs was approximately 10-fold lower than IL-4 expression in Ig.IL4 - transfected plasmacytoma cells, as measured by both a bioassay for IL-4 which measures its ability to stimulate proliferation of the HT2 indicator T cell line (Lichtman et al., Proc. Natl. Acad. Sci. USA 84:824-827, 1987), and by RNase protection analysis, as follows: RNA was isolated from cell pellets by the procedure of Chirgwin et al. (Biochemistry 18:5294-5299, 1979) using the CsCl gradient modification. RNA was dissolved in sterile distilled water and the yield determined by UV absorption at 260 nm. The antisense

RNA probe for detection of IL-4 mRNA was generated from the plasmid pGLM-1, which contains a HindIII-XmaI pBT polylinker sequence immediately 5' of a first exon IL-4 fragment from pIg.IL4. The presence of polylinker DNA in the probe allowed for the distinction between endogenous and tramsgenic IL4 mRNAs in cell lines containing the Ig.O.IL4 and Ig.O3.IL4 constructions. The β_2 microglobulin antisense RNA probe has been described (Parnes and Seidman, Cell 29:661-669, 1982). RNAase protection was carried out as described by Melton et al. (Nucl. Acids. Res. 12:7053-7056, 1984).

Using these two promoter-attenuated (operator$^+$/repressor$^-$) constructs, several transgenic founders were generated that remained viable and were capable of breeding, one (named TG.TS) with the Ig.O.IL4 construct, and three (named TG.TX, TG.UD, and TG.UG) with the Ig.03.IL4 construct. By RNAase protection analysis, mRNA for the IL-4 transgene was detected in the spleen, thymus, lymph nodes and bone marrow of all transgenic lines, indicating that the expected tissue specificity of the immunoglobulin control elements was maintained. RNA expression data for the thymus and spleen from wild-type animals and TG.UD and TG.UG heterozygates is shown in Fig. 3. No endogenous IL-4 mRNA transcripts could be detected in lymphoid tissues from either wild-type or transgenic animals. With the exception of a low level of transgene expression in the brain of TG.TS and TG.UD animals, there was no expression in non-lymphoid organs. In the thymus, the level of transgene expression (i.e., IL-4 mRNA) correlated inversely with the number of lac operator sequences inserted into the immunoglobulin promoter. Thus, the level of IL-4 transgene mRNA in the thymus was greatest in the TG.TA

founder (unattenuated construct) and was somewhat lower in the TG.TS line (single operator attenuation); expression was further reduced in the TG.TX and TG.UD (triple operator attenuation) lines, the two of which displayed comparable degrees of IL-4 transgene mRNA in the thymus. In comparison with the TG.TX and TG.UD lines, the TG.UG line, also generated from the triple operator-attenuated construct, displayed an even further (at least 10-fold) reduction in the level of expression of the IL-4 transgene in the thymus. To prepare RNA from TG.UD and TG.UG splenocyte populations enriched for B or T cells, panning on dishes coated with goat anti-mouse immunoglobulin (Southern Biotechnology Associates [SBA], Birmingham, AL) was performed (Wysocki and Sato, Proc. Natl. Acad. Sci. USA 75:2844-2848, 1978). Adherent cells were predominantly B cells, the population being >80% $B220^+$ and <10% $CD3^+$ by flow cytometric analysis (see below). Non-adherent cells were further enriched for T cells by incubation with a mixture of rat anti-mouse CD4 (GK 1.5; Dialynas et al., J. Immunol. 131:2445-2451, 1983) and rat anti-mouse CD8 (53.6.72; Ledbetter and Herzenberg, Immunol. Rev. 47:11-18, 1979) supernatants followed by plating on dishes coated with goat anti-rat immunoglobulin (SBA). A 2-3 fold enrichment of T cells compared with whole spleen was obtained; the T cell- enriched population from TG.UG, however, contained about 10-20% residual B cells. The level of expression in TG.UG splenocytes enriched for B cells was found to be significantly higher than in a T cell-enriched splenocyte population. Conversely, there was no detectable expression of IL-4 transgene mRNA from splenic B cells from the TG.UD line, but expression was clearly observed from a population enriched for T cells

(Fig. 3, lane marked "Spleen UD-T"). The differences in transgene expression in the B and T lymphocytes of TG.UD and TG.UG mice proved to be most informative with respect to understanding the phenotypes observed in these lines.

In three of the four lines studied (TG.TS, TG.TX, and TG.UD), an abnormality in thymic development similar to that of the TG.TA founder animal was observed in all transgenic offspring. Histologic analysis (Figs. 4B and 4D, respectively, show 100x and 400x magnifications of thymus sections from a TG.UD mouse) revealed the absence of a definable cortex and an increase in the population of larger lymphoid cells resembling normal medullary thymocytes. Thymic hypoplasia was also observed, with the total number of thymocytes recovered from individual animals at 4-6 weeks of age being the lowest for the TG.TS line (<10% that of littermate controls) and somewhat greater for the TG.TX and TG.UD lines (34% and 31% that of littermate controls, respectively) (Table 1). When TG.UD offspring were bred to be homozygous for the transgene, they displayed a more severe thymic hyperplasia, comparable to that of TG.TS heterozygotes, suggesting a gene dosage effect. In contrast, the thymuses of TG.UG heterozygotes were histologically normal and contained normal numbers of cells. As noted, this line differs from the affected lines in having a greatly reduced level of transgene mRNA expression in the thymus and peripheral T cells, despite a high level of expression in the B cell compartment. Thus, the thymic abnormality correlates well with the level of overexpression of IL-4 within the thymus.

To further characterize the thymic abnormality induced by IL-4 transgene expression, flow

cytometric analysis of thymic cell populations (Fig. 5) was performed, as follows: Single cell suspensions from thymuses were stained with the appropriate antibodies and analyzed by standard flow cytometry on a Cytofluorograf IIS (Ortho Diagnostic Systems Inc., Westwood, MA). A phycoerythrin-labeled (PE) antibody to mouse CD4 (GK 1.5; Becton-Dickinson, Mountain View, CA) and an FITC-labeled antibody to mouse CD8 (53.6.72; Becton-Dickinson) were used for single- and double- staining procedures. For CD3/CD8 double-staining of thymocytes, a hamster anti-mouse CD3 (500.A2; Havran et al., Nature 330:170-173, 1987) was biotinylated and followed by PE-avidin (Vector, Burlingame, CA) and FITC-labeled anti-CD8. As shown in Table 1 and Fig. 5, a marked reduction in the fraction of thymocytes coexpressing the CD4 and CD8 surface markers was seen in the TG.TS, TG.TX and TG.UD lines. $CD4^+CD8^+$ (double positive) cells, some of which are precursors to the mature single positive ($CD4^+$ or $CD8^+$) medullary thymocytes (Nikolic-Zugic and Bevan, Proc. Natl. Acad. Sci. USA 85:8633-8637, 1988; Guidos et al., Proc. Natl. Acad. Sci. USA 86:7542-7546, 1989), normally comprise 80-90% of the thymocyte population and are predominantly small lymphocytes within the cortex (Fowlkes and Pardoll, Adv. Immunol. 44:207-264, 1989). The reduction of this population therefore correlated well with the observed histologic abnormality in the affected transgenic lines. Also apparent was a great expansion of the fraction of thymocytes bearing the single positive $CD8^+$ surface phenotype (e.g., 40% for TG.TX and TG.UD heterozygotes, compared with 3% for littermate controls). Based upon a high level of expression of the CD3 surface antigen (Bluestone et al., Nature 326:82-84, 1987), as shown for the TG.UD

line (Fig. 5B), the expanded $CD8^+$ population appeared to represent mature thymocytes. The expression of the CD3 antigen distinguishes the mature $CD8^+$ population from a population of immature $CD3^-CD4^-CD8^+$ thymocytes which are precursors to cortical $CD4^+CD8^+$ cells (Fowlkes and Pardoll, 1989). These mature $CD8^+$ cells could also be distinguished on the basis of their larger size, compared with the majority of normal thymocytes, resulting in a bimodal distribution of thymocyte size (Fig. 5B, "size" profile). The expansion of the number of larger cells was again consistent with the histologic findings. The high level of surface CD3 expression on the majority of $CD8^+$ thymocytes was readily apparent when the population of large cells was analyzed independently (Fig. 5C). Furthermore, when this population was excluded from the surface phenotype analysis, the remaining population exhibited normal CD4/CD8 and CD3/CD8 profiles (Fig. 5D). In terms of absolute cell number, the population of mature $CD8^+$ thymocytes was increased 4-fold over controls in TG.TX and TG.UD heterozygotes, along with an 8-fold reduction in the $CD4^+CD8^+$ population. $CD4^-CD8^-$ thymocytes, the majority of which in the normal thymus represent an early precursor population (Fowlkes et al., J. Exp. Med. 162:802-822, 1985), were maintained or somewhat increased in absolute number in these lines. As expected, the histologically-normal thymus of TG.UG mice displayed no abnormalities by flow cytometric analysis (Fig. 5E).

Neutralizing monoclonal anti-IL-4 antibody (11B11; Ohara and Paul, Nature 315:333-336, 1985) was prepared from ascites fluid, as previously described (Tepper et al., Cell 57:503-512, 1989), yielding a final protein concentration of approximately 16

mg/ml. The effect of combined in utero (delivered via the maternal circulation) and neonatal administration of large doses of the anti-IL-4 monoclonal antibody was determined by the following methodology: Antibody (0.5 cc) was administered by intraperitoneal (i.p.) injection to a female TG.UD heterozygote on the day following mating to a wild-type FVB/N male and at weekly intervals until delivery. Beginning on postnatal day 5, 0.1 cc of antibody was administered weekly by the i.p. route to individual pups until they were sacrificed for analysis at four weeks of age. It was found that such combined in utero and neonatal treatment could restore toward normal the number and surface phenotype of thymocytes in TG.UD mice (Table 1). This evidence further supports a causal relationship between the aberrant expression of the IL-4 gene product and the abnormality observed. The transplantation (by intravenous injection) of 10^7 bone marrow cells from 4-week-old wild-type male FVB/N mice into irradiated (750 rads) 4-week-old female TG.UD heterozygotes (with chimerism confirmed by the presence of Y chromosomal DNA sequences upon Southern blot analysis of spleen DNA using the probe pY2 (Lamar and Palmer, Cell 37:171-177, 1984) resulted in the appearance, when analyzed at four weeks following bone marrow injection, of a histologically-normal thymus and the complete restoration of a normal CD4/CD8 profile (Fig. 4F and Table 1). Conversely, the transfer of bone marrow cells from a TG.UD heterozygote male into an irradiated wild-type FVB/N female recipient established the characteristic histologic abnormality and abnormal cytometric profile of the TG.UD transgenic animal (Fig. 4E and Table 1). Taken together, these data indicated that the thymic defect was a result of abnormal expression of

IL-4 within developing thymocytes, rather than a primary alteration in the radioresistant thymic epithelium or other stromal components.

In normal mice systemically treated with corticosteroids, the $CD4^+CD8^+$ thymocyte population is depleted, while mature single positive thymocyte populations are maintained (Blomgren and Anderson, Cell Immunol. 1:545-560, 1971). While it may be hypothesized that hypercortisolemia, as a physiologic response to severe infections (Sapolsky et al., Science 238:522-524, 1987), could explain the reduction in $CD4^+CD8^+$ thymocytes observed in IL-4 transgenic mice, there was no evidence for increased susceptibility to infection or shortened survival in TG.TX or TG.UD heterozygotes, both of which display the thymic abnormality. Furthermore, the alteration in thymocyte composition as a result of IL-4 expression in IL-4 transgenic lines differed from the effects of corticosteroids in that the $CD8^+$ population was selectively expanded. A corticosteroid- mediated effect is therefore unlikely to account for the changes in thymocyte populations observed in IL-4 transgenic mice. A reduction in the $CD4^+CD8^+$ population and an expansion of both the mature $CD4^+$ and $CD8^+$ populations of the thymus has also been reported in transgenic mice that overexpress c-FOS in the thymic epithelium from the $H2\text{-}K^b$ promoter (Ruether et al., Cell 53:847-856, 1988). In contrast to the latter mice, increased numbers of thymic epithelial cells are not observed in the IL-4 transgenic animals, nor does the level of thymic c-fos expression appear to be altered in IL-4 transgenic animals.

IL-4 in combination with phorbol myristate acetate (PMA) has been shown to induce the in vitro proliferation of adult $CD8^+$ and (to a lesser extent)

$CD4^+$ thymocyte populations, $CD4^+CD8^+$ cells being unaffected (Zlotnick et al., Proc. Natl. Acad. Sci. USA 84:3856-3860, 1987). The $CD4^-CD8^-$ population from both adult (Zlotnick et al., 1987) and day 15 fetal (Zlotnick et al., 1987; Palacios et al., EMBO J. 6:91-95, 1987) thymus also responds to IL-4 in vitro by proliferation and differentiation to mature $CD8^+$ cells; again, however, PMA is required. Moreover, $CD4^-CD8^-$ thymocytes can produce IL-4 in vitro when stimulated with the calcium ionophore A23187 plus PMA (Zlotnick et al., 1987; Ransom et al., J. Immunol. 139:4102-4108, 1987). By in situ hybridization analysis, IL-4 mRNA can be demonstrated in 5% of unstimulated day 13 and almost 50% of unstimulated day 15 fetal thymocytes, but not at other times during fetal development; interleukin 2 ("IL-2") expression follows the same temporal distribution (Carding et al., Proc. Natl. Acad. Sci. USA 86:3342-3345, 1989). Although the precise precursor-progeny relationships between IL-4-producing immature thymocytes and more mature cells remains to be defined, these in vitro and in situ results provide indirect evidence of a role for IL-4 in intrathymic T cell development, in agreement with the ability, observed in the IL-4 transgenic mice of the invantion, of IL-4 to affect thymic development, independent of exogenous activating factors, when acting within the context of the in vivo thymic microenvironment. IL-4 may act selectively to expand a mature $CD8^+$ thymocyte population by promoting maturation of precursors or, alternatively, by inducing proliferation of differentiated $CD8^+$ cells. In addition, the marked reduction in $CD4^+CD8^+$ thymocytes, the severity of which reduction varies directly with the level of IL-

4 expression, may normally play a role in intrathymic cell death, a physiologic process which occurs during the $CD4^+CD8^+$ stage of thymic differentiation (Rothenberg and Lugo, Dev. Biol. 112:1-17, 1985).

A reduction in the T cell population of the spleen was observed in IL-4 transgenic mice, as determined by flow cytometry (Table 2) using the following antibodies against lymphocyte cell surface markers: unconjugated rat antibodies to mouse B220 (6B2; Coffman, Immunol. Rev. 69:5-23, 1982), Thy-1 (M5/49; Davignon et al., Proc. Natl. Acad. Sci. USA 78:4535-4539, 1981) and CD3 (C363.29B, provided by Dr. K. Bottomly) were followed by a fluorescein-labeled (FITC) goat anti-rat IgG (Kirkegaard & Perry, Gaithersburg, MD); FITC-labeled goat anti-mouse kappa (SBA) was used for kappa light chain detection. In addition, a PE-labeled antibody to mouse CD4 and an FITC-labeled antibody to mouse CD8 were used for single- and double-staining procedures. The severity of the T cell deficiency correlated with the degree of thymic hypoplasia, with TG.TS heterozygotes and TG.UD homozygotes being most deficient and TG.UG heterozygotes displaying no T cell reduction, despite a high level of IL-4 transgene expression in the spleens of these animals.

In addition, the proliferative response of spleen cells to concanavalin A (Con A, Sigma), a T cell mitogen, was determined as follows: splenocytes from four IL-4 transgenic lines and from wild-type controls were cultured at $0.25\text{-}4\times10^5$ cells per well in 0.2 ml RPMI 1640 medium supplemented as described above, using Con A at a final concentration of 2.5 μg/ml. Cultures were performed in flat-bottom microculture plates at 37°C in 5% humidified CO_2 for 72 hr. For the last 6-8 hr, each well was pulsed with

1μCi of [^{3}H]- thymidine (Amersham). Cultures were harvested on a PHD automated sample harvester (Cambridge Technology, Cambridge, MA), and incorporated radioactivity was measured by scintillation counting. The results of Con A stimulation, shown in Fig. 6, demonstrate a deficiency in functional T cells in transgenic mice which was again proportional to the degree of thymic hypoplasia. Consistent with these findings was the observation that susceptibility to viral infections (e.g., mouse hepatitis virus) was increased in homozygotes from the TG.UD line and in TG.TS heterozygotes, but not in TG.UD or TG.TX heterozygotes. With TG.UD heterozygotes (and to a lesser extent TG.TX heterozygotes), the deficient Con A response could be restored by increasing the number of responding cells (e.g., in Fig. 6, compare the response of $1x10^5$ control spleen cells to $4x10^5$ TG.UD spleen cells), suggesting that the T cell defect was predominantly quantitative. Spleen cells from the T cell-deficient transgenic mice also showed reduced proliferative responses to immobilized anti-CD3 antibody and PMA plus ionomycin, which are thought to mimic physiologic T cell activation (Weiss and Imboden, Adv. Immunol. 41:1-38, 1987), and to IL-2.

Furthermore, in response to Con A or anti-CD3, splenocytes from TG.UD heterozygotes secreted reduced levels of IL-2, commensurate with the observed reduction in the splenic T cell population. Despite the presence of mRNA for the IL-4 transgene in both uninduced and Con A-induced spleen cells from this line (Fig. 3B; spleen UD TOT and spleen UD CON A lanes), IL-4 activity was not detectable in culture supernatants from these populations by bioassays using two IL-4 responsive T cell lines, HT2 and CT4S, which

are sensitive to IL-4 concentrations of 10U/ml or greater. The level of transgenic IL-4 mRNA was significantly lower than the level of endogenous IL-4 mRNA observed with Con A- induced wild-type spleen cells or transgenic IL-4 mRNA from uninduced TG.UG spleen or TG.UG splenic B cells (Fig. 3B; spleen WT CON A, UG TOT, and UG B lanes), signals also not detectable by bioassay. The failure to detect secretion of biologically active IL-4 in vitro may be due to the insensitivity of the bioassay or a result of the absorption of IL-4 to the surface of cultured splenocytes.

Of note, the $CD8^+$ fraction of splenic T cells appeared to be more severely reduced than the $CD4^+$ fraction in the T cell-deficient IL-4 transgenic mice (Table 2). If IL-4 plays a role in the directional maturation of precursor thymocytes to mature $CD8^+$ cells, as suggested by the IL-4 transgenic strains of the invention, additional signals must be required to promote their egress to the periphery. It is also possible that IL-4 itself may interfere with the normal process of egress from the thymus, the signals for which are poorly understood.

In addition to the deficiency in peripheral T cells, a single transgenic line, TG.TS, displayed a marked reduction in the B lymphocyte population of the spleen, as demonstrated by the lack of cell surface staining for B220 and kappa light chain (Table 2) and by an observed failure of spleen cells to proliferate in response to lipopolysaccharide (LPS), a polyclonal B cell mitogen. Marked lymphoid depletion in the spleen was noted histologically in this line, and as a result of the combined T and B cell immunodeficiency, early death (by six weeks of age) due to infection was observed. Although the basis for the deficiency is

unclear, it is noteworthy that B cell reduction is observed only in the line with the most severe T cell deficiency. This raises the possibility that normal B cell development might be interrupted by the absence of critical cellular interactions or helper cytokines provided by T cell populations.

In the immunocompetent TG.UG line, the high level of constitutive IL-4 expression in the spleen resulted in marked splenomegaly (3-4 fold increase by weight compared with littermate controls) with expansion of both B and T lymphocyte populations as well as non-lymphoid cells. A lesser degree of splenic hyperplasia was also observed in the TG.UD and TG.TX lines. The development of comparable degrees of splenic hyperplasia (2-4 fold increase by weight and cell number) can be induced in wild-type animals within 72-96 hours by the continuous systemic infusion of high doses of purified recombinant IL-4. These findings suggest that one mechanism of splenic enlargement as observed in various infectious and inflammatory states may be the elaboration of T cell-derived cytokines such as IL-4.

In order to assess the effect of constitutive IL-4 expression on the production of IgE and IgG immunoglobulin isotypes, ELISA determinations on serum samples from mice in each of the transgenic lines were performed, as follows: serial dilutions of serum from 1:100 to 1:500 were added to microculture wells coated with 10 μg/ml of purified rat anti-mouse monoclonal antibody, EM 95 (Baniyash and Eshhar, Eur. J. Immunol. 14:799-807, 1984), incubated at room temperature for 90-120 min, and washed. Biotin-conjugated goat anti-mouse IgE at a concentration of 2 μg/ml was then added for 2 hr at room temperature,

followed by avidin-conjugated alkaline phosphatase (SBA). After washing, enzymatic activity was developed using the substrate $_{\pi}$-nitrophenyl phosphate disodium (Sigma), and optical density at 405 nm was determined in an ELISA reader (Bio-Rad). Serum levels were calculated from a standard curve using a purified mouse monoclonal IgE (ATCC # TIB142; Rudolph et al., Eur. J. Immunol. 11:527-529, 1981). Serum IgG1 and IgG2a levels were measured as described (Boom et al, J. Exp. Med. 167: 1350-1363, 1988), using serum dilutions of 1:500 to 1:5000, with mouse monoclonal antibodies as standards. As shown in Fig. 7A, marked elevations in serum IgE levels were noted for all IL-4 transgenic lines studied, the levels in TG.UD homozygotes and TG.UG heterozygotes being at least 20-25 fold larger than the levels in non-transgenic littermates. In TG.UD homozygotes, IgE accounted for over 8% of the total serum immunoglobulin, compared with less than 0.2% for wild-type controls. IgG isotype analysis also revealed increased serum levels of IgG1 and a reduction in IgG2a in the TG.UD and TG.UG lines (Fig. 7B). IL-4 has been shown to enhance the cell surface expression and secretion specifically of IgG1 and IgE <u>in vitro</u> by LPS-stimulated B cells (Vitetta et al., J. Exp. Med. 162:1726-1730, 1985; Coffman et al., J. Immunol. 136:949-954, 1986) through the induction of immunoglobulin heavy-chain class switching (Snapper and Paul, Science 236:944-947, 1987; Lutzker et al., Cell 53:177-184, 1988). Moreover, its role as a physiologic regulator of IgE production has been suggested by studies demonstrating that anti-IL-4 antibody inhibits the <u>in vivo</u> IgE response to helminthic parasites or anti-IgD antibody (Finkelman et al. J. Immunol. 141:2335-2341, 1988a). The data disclosed herein provide further evidence

that IL-4 can act in vivo to direct immunoglobulin synthesis to the production of IgE and IgG1.

Of interest is the association of several human T cell immunodeficiency states with the excessive production of IgE antibody: namely, the Wiskott-Aldrich syndrome, the Nezelof syndrome, and the hyperimmunoglobulin E (Job's) syndrome (Buckley, Immunologic Deficiency and Allergic Disease. In Allergy: Principles and Practice, Vol. 1, E. Middleton, Jr., ed.; C.W. Mosby Co., 1988). It has been postulated that alterations in the balance between specific T cell- derived lymphokines promoting IgE production, such as IL-4, and negative regulators for the production of this isotype, such as interferon-γ (Coffman and Carty, J. Immunol. 136:949-954, 1986; Finkelman et al., J. Immunol. 140:1022-1027, 1988b), may play a role in the immunoglobulin aberrations seen in these disorders (Geha and Leung, Immunodef. Reg. 1:155-172, 1989; Buckley, 1988). Moreover, the high incidence of allergic disease in these disorders and the general association of excessive IgE production with allergy in human subjects (Buckley and Becker, Immunol. Rev. 41:288-314, 1978) may be particularly relevant with regard to an inflammatory condition noted in the IL-4 transgenic lines, as discussed below.

In each IL-4 transgenic line displaying the thymic abnormality (TG.TS, TG.TX, and TG.UD; but not TG.UG), a striking inflammatory lesion of the external eye was noted, characterized grossly by marked swelling and erythema of the eyelid (Fig. 8A, showing a homozygous TG.UD transgenic mouse on the right and a wild-type FVB/N mouse on the left). Significantly, the severity and frequency of the lesion correlated directly with the level of transgene expression within

the T cell compartment. Thus, the abnormality was bilateral and present in 12/12 TG.TS heterozygous and 5/5 TG.UD homozygous animals examined, while in TG.UD and TG.TX heterozygotes the lesion was present in 35% of over 50 transgenic offspring studied, and was typically unilateral.

Histologic analysis of the eyelid lesion was carried out as follows: tissues were fixed in phosphate- buffered formalin, blocked in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (Fig. 8B), or toluidine blue (Fig. 8C). Microscopic analysis of the lesion tissues revealed a dense inflammatory infiltrate involving the subepithelial stroma, and composed of mononuclear cells and a striking number of eosinophils (Fig. 8B). Toluidine blue staining also demonstrated the presence of an excessive number of tissue mast cells in a lesion from a TG.UD mouse (Fig. 8C). The involvement of mast cells and eosinophils in a number of inflammatory reactions associated with allergy in humans is well established (Holgate et al., Mediators of Immediate Hypersensitivity. In Allergy: Principles and Practice, Vol. 1, E. Middleton, Jr., ed.; C.W. Mosby Co., 1988). While the cellular infiltrate observed could conceivably be due to a normal response against an infectious agent [for example, an helminthic parasite (Butterworth, Adv. Parasitol. 23:143-235, 1984)], the lack of an histologically-identifiable pathogen, the presence of the lesion in animals maintained in an pathogen-free isolation facility, and the lack of horizontal transmission of the disorder to wild-type or unaffected transgenic littermates argue against an infectious etiology.

The marked predisposition of IL-4 transgenic mice to an allergic-like disorder is intriguing, given a number of the activities ascribed to IL-4. First, as we have observed in this study, IL-4 overexpression in the lymphoid compartment in vivo is associated with the hypersecretion of IgE. As noted, a role for IL-4 in the induction of human IgE is also supported by in vitro studies (DelPrete et al., J. Immunol. 140:4193-4198, 1988; Pene et al., Proc. Natl. Acad. Sci. USA 85:6880- 6884, 1988). In humans, IgE plays an important role in the initiation of allergic reactions by interacting with the high-affinity Fcε receptor (FcεRI) on mast cells and basophils (Metzger et al., Prog. Immunol. 5:493-501, 1983; Conrad et al., J. Immunol. 130:327-333, 1983). Upon cross-linking of IgE-bound Fc receptors, these effector cells release chemotactic and inflammatory mediators. IL-4 has also been shown to enhance the expression of the low-affinity receptor for IgE (FcεRII, CD23) on human B lymphocytes and monocytes (Kikutani et al., Cell 47:657-665, 1986; Defrance et al., J. Exp. Med. 165:1459-1467, 1987; Vercelli et al., J. Exp. Med. 167:1406-1416, 1988) and induces a specific isoform (FcεRIIb) on these cells (Yokota et al., Cell 55:611-618, 1988). The enhanced expression of FcεRII on B lymphocytes of allergic patients (Spiegelberg et al., 1979; Suemura et al., J. Immunol. 137:1214-1220, 1986), and the abnormal, constitutive expression of the FcεRIIb isoform in a patient with allergic disease (Yokota et al., Cell 55:611-618, 1988) have been reported. IL-4 in conjunction with interleukin 3 ("IL-3") has been shown to stimulate the in vitro proliferation of mast cell lines (Lee et al., Proc. Natl. Acad. Sci. USA 83:2061-2065, 1986; Mosmann et al.,

Proc. Natl. Acad. Sci. USA 83:5654-5658, 1986; Hamaguchi et al., J. Exp. Med. 165:268-273, 1987). The presence of increased numbers of mast cells in the ocular lesions of transgenic mice in this study suggests that this activity of IL-4 is of significance in vivo. Furthermore, it has been previously demonstrated that localized expression of IL-4 in vivo can promote eosinophil chemotaxis (Tepper et al., 1989).

Taken together, the above findings suggest that IL-4 deregulation may be central to the pathogenesis of the allergic state. With regard to the IL-4 transgenic mice, it is of interest that the ocular inflammatory lesion was not observed in the TG.UG line, despite a marked elevation in serum IgE levels. As noted, the TG.UG line is distinct in that it displayed no thymic abnormality or peripheral T cell deficiency, with expression of the transgene being predominantly within the B lymphocyte lineage. This suggests that deregulated expression of IL-4 within cells of the T lymphocyte lineage may be necessary to induce the allergic-like phenotype. It has been recently observed that the frequency of IL-4-producing T cell clones derived from the conjunctiva of patients with vernal conjunctivitis, an ocular allergic disorder, is greatly increased in comparison with controls (S. Romagnani, in press). It is possible that the elaboration of IL-4 by T lymphocytes present locally at the site of the lesion serves to incite the characteristic inflammatory infiltrate seen in IL-4 transgenic mice. Alternatively, specific alterations in peripheral T cell populations (e.g., the preferential reduction of the $CD8^+$ lymphocyte population) observed in these lines as a result of the

IL-4-induced perturbation in thymic development may contribute to the observed phenotype.

Since IL-4 is normally produced by a subset of $CD4^+$ T lymphocytes, its deregulated expression within the T lymphocyte lineage in several of the transgenic mouse lines of the invention may have particular relevance to the understanding of its actions in both physiologic and pathologic host responses and in the ontogeny of T cells. The notion that the specific types of humoral and cellular effectors elicited in response to an antigenic challenge may depend on the preferential production of specific lymphokines (Mosmann and Coffman, Ann. Rev. Immunol. 7:145-173, 1989) is testable by the construction of transgenic strains analogous to the IL-4-overexpressing strains of the invention.

Example 3: $Operator^+/Repressor^+$ Transgenic Mice

In order to test the feasibility of an $operator^+/repressor^+$ expression-control system prior to inserting it into animals, preliminary experiments were conducted in which a first gene encoding the lac repressor protein, and a second gene encoding a hormone [IL-4 or human growth hormone (GH)] and bearing a lac operator sequence inserted into its promoter, were transfected into cultured cells; expression of the hormone gene in the transfected cells was measured in the presence and absence of IPTG, an inactivator of the lac repressor. In one such set of experiments, J558L plasmacytoma cells were transiently transfected with one of the following plasmids or pairs of plasmids, each of which plasmids bears one of the genes is illustrated in Fig. 9: pIgIL4; pIgEPOIL-4 (bearing the Ig.O.IL-4 gene, which includes one copy of the lac O sequence); pIgEPIA

(bearing the mouse immunoglobulin heavy chain enhancer, the human immunoglobulin heavy chain promoter, and a gene encoding the E. coli lac repressor protein linked to the SV40 polyA site); pIgEPIAneo (identical to pIgEPIA except that 3' to the SV40 polyA-encoding sequence and in opposite orientation is a gene encoding neomycin resistance, expression of which is driven from the SV40 early promoter); pIgEPOIL-4 plus pIgEPIA; pIgEPOIL-4 plus pIgEPIAneo; pIgIL4 plus pIgEPIA; and pIgIL4 plus pIgEPIAneo. Transient transfection was accomplished by electroporation (Potter, Proc. Natl. Acad. Sci. USA 81:7161-7165, 1984). Following incubation of the transfected cells in culture medium for approximately 48 hours, the medium from each set of transfected cells was assayed for IL-4 by measuring the ability of the medium to stimulate ^{3}H-thymidine uptake by HT2 cells, the proliferation of which has been shown to be stimulated by the presence of IL-4 (Lichtman et al., 1987). The results of these in vitro experiments, set forth in Table 3, indicate that (a) the presence of an operator sequence in the promoter reduces the level of expression from the promoter; (b) the presence of a lac repressor plasmid reduces expression from a lac operator- containing promoter but not from a promoter which does not contain a lac operator sequence; and (c) repression of expression by the lac repressor is dose-dependent: i.e., an increase in the molar ratio of lac repressor plasmid (pIGEPIA) relative to operator^{+} IL-4 plasmid (pIGEPOIL-4) results in a significant decrease in the level of expression of the IL-4 gene.

Stable transfection of J558L cells with both pIgEPIAneo and pIGEPOIL-4 resulted in identification of three separate clones of cells testing positive for

both neomycin resistance and IL-4 secretion into the medium, as measured by the HT2 cell proliferation assay. Upon incubation of each of these transfected J558L clones in the presence of 10mM IPTG, the level of IL-4 secreted by each clone increased by more than six-fold (Table 4). These results suggest that lac repressor is expressed within the stably transfected cells, that it acts upon the lac operator sequence in the IL-4 promoter to repress expression of the IL-4 gene, and that IPTG can enter the cells and reverse this repression.

The lac operator$^+$/repressor$^+$ method of controlling expression was also tested in a different in vitro system, using murine erythroleukemia (MEL) cells tranfected with (a) a lac repressor gene driven by a friend leukemia virus (FLV) promoter, and (b) a GH gene driven by an FLV promoter into which had been inserted one, two, or three lac O sequences. The relative amounts of GH secreted by clones of these transfected MEL cells, both in the presence and absence of IPTG, was determined by the use of a radiolabelled antibody sandwich assay specific for GH. By means of this assay, it was determined that (i) two or three lac O sequences present in a single promoter result in a lower level of expression from that promoter than from a similar promoter having only one lac O sequence, and (ii) IPTG significantly derepresses GH secretion in each of these clones (Table 5).

These results fully demonstrated the functionality of an operator$^+$/repressor$^+$ expression control system in cell lines cultivated in vitro. The next step was to generate a transgenic animal bearing a gene for the lac repressor protein, accomplished by microinjecting a Sph-I/BamHI fragment of pIgEPIA into

fertilized FVB/N mouse oocytes. Of those microinjections which resulted in viable embryos, nine of the resulting mouse lines were tested for the presence of lac repressor mRNA by RNase protection analysis, and one of the lines so tested was found to produce detectable levels of lac repressor protein in at least one tissue. In this repressor$^+$ transgenic mouse line, termed "TH", very low levels of lac repressor mRNA were found in the thymus, mesentheric lymphnode, spleen, and lung, while somwhat higher levels were detected in brain tissue. No physical abnormalities were noted upon gross or microscopic examination of these repressor$^+$ mice, all of which appear to have a normal lifespan and reproductive capacity.

A TH (repressor$^+$) transgenic mouse was sexually crossed with a TX (operator$^+$) transgenic mouse, which constitutively expressed IL-4 from an Ig promoter into which one copy of the lac O sequence has been inserted. Offspring of this mating were found to distribute among four genotypic classes (operator$^+$/repressor$^+$, operator$^+$/repressor$^-$, operator$^-$/repressor$^+$, operator$^-$/repressor$^-$), as expected if the two transgenes originally inserted on different chromosomes. The level of transgenic IL-4 mRNA produced in the brains of mice from two of these classes, the operator$^+$/repressor$^+$ class and the operator$^+$/ repressor$^-$ class, were compared by RNase protection analysis and found to be at comparably low levels near the lower limits of detectability. Brain was the organ studied because it was the site of highest repressor expression; however, as transgenic IL-4 expression in brain tissue is relatively low even in the absence of the repressor protein (i.e., in TX mice or their operator$^+$/repressor$^-$ offspring), this

was not an ideal tissue in which to study repression of transgenic inconclusive results obtained are attributable to one or a combination of the following: (a) the low level of transgenic IL-4 produced in brain tissue of even the repressor$^-$ mice; (b) the low level of lac repressor protein produced in the repressor$^+$ mouse strain; and (c) the inability of the repressor protein, which is synthesized in the cytoplasm, to penetrate the nuclear membrane and thereby reach the operator sequence on the genomic DNA. These problems will be overcome by either of two methods: (i) repeating the microinjection experiments to create new strains expressing higher levels of repressor protein in tissues in which transgenic IL-4 is produced at a high level, such as thymus, or (ii) generating new repressor$^+$ transgenic mice by microinjecting into oocytes a lac repressor gene which has been engineered to encode a nuclear translocation signal sequence, such as the Pro_{126}- Pro_{135} segment of the SV40 large T protein (Kalderon et al., Nature 311:33-38, 1984); the 13-residue amino terminal sequence of the S.cerevisiae α2 protein (shown in Fig. 10; see Hall et al., Cell 36:1057-1065, 1984); or the "tail" fragment of X.laevis nucleoplasmin (Dingwall et al., Cell 30:440-458, 1982), attached to the amino terminus of the lac repressor protein. Such a nuclear translocation signal sequence would be expected to transport the repressor protein into the nucleus of the cell where it can act on the operator sequence.

Use

The IL-4-overexpressing transgenic animals of the invention are useful as animal models for certain immunological abnormalities, to test proposed treatments for human diseases characterized by such

abnormalities. In particular, those animals of the invention which exhibit a heightened allergic response, including the tendency to develop an allergy-like inflammatory lesion of the eyelid, can be used to test proposed treatments for human allergies.

The two expression-attenuating methods of the invention can be applied to the generation of many different types of transgenic animals expressing any given transgene in virtually any tissue, where expression in the absence of such expression-attenuation is higher than is desired, or where experimental control over the level of expression at any given time is desired. These methods are particularly useful where unattenuated expression of the transgene is lethal to the transgenic animal, or otherwise prevents the animal from being able to reproduce and thus maintain the transgenic germline. The operator$^+$/repressor$^+$ system of the invention, expression from which can be regulated at will by the use of a repressor inactivator (such as IPTG), offers fine-tuned temporal control of expression of the transgene. These particular animals can be used in studies on the effect of the transgene protein product on, for example, stages of embryological development, behavior, cancer etiology and growth, and the immunological response.

Other Embodiments

Other embodiments are within the following claims. For example, the methods of generating an operator$^+$/repressor$^-$ or operator$^+$/repressor$^+$ transgenic animal set forth in Examples 2 and 3, respectively, can be applied not only to a gene encoding murine IL-4, but also to any other

transgene. Examples of likely candidate genes include those encoding other interleukins, other hormones, regulatory factors, neurotransmitters, enzymes, structural proteins, viral proteins, and oncogenes. The operator$^+$/repressor$^+$ system utilized can be that of the E. coli lac operon, as illustrated in Example 4, or can alternatively be a different operator/repressor system, such as that of bacteriophage λ, bacteriophage 434, bacteriophage P22, or the E.coli Trp operon. Minor changes in the operator or repressor sequences which do not interfere with repressor control of transcription are within the invention. The means of reversing repression will differ according to the operator/repressor system used, with IPTG or other stable analogs of lactose being useful with the lac operator/repressor system and other entities being useful with other systems. The identities of repressor inhibitors for each known operator/repressor system are well known in the art, or can be developed and tested by known means. Likewise, the identity of the "operator" in the operator$^+$/ repressor$^-$ system may differ from the particular DNA segment utilized in Example 3, the lac operator. Some of the potential substitutes for the lac operator would include the operator of the E. coli tet operon, the E. coli met operon, and the E. coli gal operon; the phage lambda operator; the phage 434 operator; the phage 21 operator; the phage 22 operator; the yeast STE6 operator; the dyad symmetry element of the human c-fos promoter; the AP-1 transcription factor binding site; and the estrogen receptor binding site. Alternatively, the "operator" may be, rather than a true operator or protein-binding site derived from a natural gene, a DNA sequence which is heterologous to (i.e., does not occur naturally

within) the promoter region, and which contains a palindromic or dyad-symmetry sequence, types of DNA sequences frequently found in these natural operators and protein binding sites. It is thought that the operator$^+$/repressor$^-$ system reduces the level of transcription by making the binding of RNA polymerase to the promoter less efficient, whether by providing a site to which endogenous DNA-binding factors will serendipitously bind and thereby interfere with binding by RNA polymerase, or by introducing DNA secondary structure that interferes with transcription. Therefore, many different heterologous DNA segments having palindromic or dyad-symmetry character may be used in the operator$^+$/repressor$^-$ method of the invention. The sequence utilized, whether a natural operator or otherwise, should contain at least 6 and up to 100 base pairs, with 8-50 base pairs being a useful range and 10-30 being preferred. Each heterologous DNA segment can be tested for ability to attenuate expression by synthesizing it, incorporating it into a promoter or other untranslated part of the gene of interest, and generating transgenic animals bearing that "operator$^+$" gene. The sequence can be present as a single copy in the transgene, or can be two or more copies which are linked or separate, or even which differ from one another in length and/or sequence. The number of copies of this heterologous DNA segment present in a given untranslated region determines the degree of attenuation of transcription of that gene, so that in some cases a series of transgenic animal lines, such segment, would be useful. Although in theory any number of such heterologous DNA segments may be used in a given transgene, in practice ten copies is the upper limit for optimal attenuation. The heterologous

DNA segments can be inserted into any portion or portions of the promoter region, from the 5' end of the promoter (e.g., within 1,000 bp to the 5' side of the so-called "TATA box") to the transcription start site, and can be adjacent to one another or spaced apart within the promoter. Alternatively, the heterologous DNA sements could be inserted anywhere in the 5' untranslated region (between the transcription start site and the translation initiation codon); in an intron; or even, by careful manipulation of redundant codons in a given gene to produce, for example, a exon sequence that is palindromic (or has dyad symmetry) and still encodes the same protein. The latter manipulation could be accomplished by a computer search of the transgene exon sequences for appropriate adjacent codons that, when substituted with other codons encoding the same residues as the original codons, form, for example, a palindromic sequence.

Similarly, the invention includes not only the particular strains of IL-4-transgenic mice disclosed herein, but also other transgenic mouse strains or any other transgenic non-human vertebrate animals, which are transgenic for murine IL-4 or heterologs of murine IL-4 (including the IL-4 of any mammalian species, such as human). The promoter region attached to a particular IL-4-transgene can be any promoter sequence other than the one which naturally occurs immediately 5' to the transcription start site for that IL-4 gene (i.e., any heterologous promoter region). Examples of useful promoter regions include mammalian immunoglobulin promoters (whether attached to mammalian enhancer regions or not); promoters for the actin family of genes; the mammalian CD-2, Thy-1, elastase, c-fos, or metallothionein

promoter; the long-terminal repeat of MMTV; or the SV40 early-region promoter. Selection of a particular promoter region will depend upon the type of tissue in which expression is to be targeted, with immunoglobulin promoters, such as the immunoglobulin heavy chain promoter, useful for targeting lymphoid tissues such as thymus and spleen.

What is claimed is:

Claims

1. A transgenic non-human vertebrate animal having cells containing a transgene encoding IL-4, which transgene was introduced into said animal, or an ancestor of said animal, at an embryonic stage.

2. The animal of claim 1, wherein said transgene comprises a heterologous promoter region.

3. The animal of claim 2, wherein said heterologous promoter region does not comprise the naturally-occurring IL-4 promoter.

4. The animal of claim 2, wherein said heterologous promoter region comprises a promoter selected from the group consisting of a mammalian immunoglobulin promoter; promoters for the actin family of genes; the mammalian CD-2, Thy-1, elastase, c-fos, or metallothionein promoter; the long-terminal repeat of MMTV; or the SV40 early-region promoter.

5. The animal of claim 4, wherein said heterologous promoter region comprises a mammalian immunoglobulin enhancer and promoter.

6. The animal of claim 1, wherein said transgene is expressed predominantly in lymphoid tissues of said animal.

7. The animal of claim 1, wherein said animal exhibits a heightened allergic response compared to wild-type animals of the same species.

8. The animal of claim 1, wherein said animal is predisposed to develop an inflammatory lesion of the eyelid.

9. A transgenic non-human vertebrate animal having cells containing a transgene having an untranslated region comprising a heterologous DNA segment comprising 6 base pairs, said heterologous DNA segment being selected from a group consisting of an operator, a eukaryotic transcription factor binding site, a palindromic sequence, or a sequence having dyad symmetry, which transgene was introduced into said animal, or an ancestor of said animal, at an embryonic stage.

10. The animal of claim 9, wherein said heterologous DNA segment is selected from a group consisting of the operators of the E. coli lac operon, the E. coli tet operon, the E. coli met operon, and the E. coli gal operon; the phage lambda operator; the phage 434 operator; the phage 21 operator; the phage 22 operator; the yeast STE6 operator; the dyad symmetry element of the human c-fos promoter; the AP-1 transcription factor binding site; the estrogen receptor binding site; a palindromic sequence of 8-50 base pairs; and a sequence of 8-50 base pairs having dyad symmetry.

11. The animal of claim 10, wherein said heterologous DNA segment comprises the operator of the E. coli lac operon.

12. The animal of claim 10, wherein said heterologous DNA segment comprises the following palindromic sequence:

ATTGTGAGCGCTCACAAT
TAACACTCGCGAGTGTTA.

13. The animal of claim 9, wherein said untranslated region comprises one to ten copies of said heterologous DNA segment.

14. A transgenic non-human vertebrate animal having cells containing a transgene encoding a heterologous repressor protein, which transgene was introduced into said animal, or an ancestor of said animal, at an embryonic stage.

15. The animal of claim 14, wherein said heterologous repressor protein comprises the E. coli lac repressor protein.

16. The animal of claim 14, wherein said cells further contain a second transgene comprising an operator sequence to which said repressor protein is capable of binding.

17. The animal of claim 1, claim 9, or claim 14, wherein said animal is a mammal.

18. The animal of claim 17, wherein said mammal is a rodent.

19. The animal of claim 1, claim 9, or claim 14, wherein said cells comprise somatic cells and germ line cells.

20. A method of making a transgenic non-human vertebrate animal having cells containing a transgene, the level of expression of which is

attenuated by the presence, in the untranslated region of said transgene, of a heterologous DNA segment having 6 to 100 base pairs, said heterologous DNA segment being selected from a group consisting of an operator sequence, a eukaryotic transcription factor binding site, a palindromic sequence, or a sequence having dyad symmetry, which transgene was introduced into said animal, or an ancestor of said animal, at an embryonic stage.

21. The method of claim 19, wherein said heterologous DNA segment is selected from a group consisting of the operators of the E. coli lac operon, the E. coli tet operon, the E. coli met operon, and the E. coli gal operon; the phage lambda operator; the phage 434 operator; the phage 21 operator; the phage 22 operator; the yeast STE6 operator; the dyad symmetry element of the human c-fos promoter; the AP-1 transcription factor binding site; the estrogen receptor binding site; a palindromic sequence of 8-50 base pairs; and a sequence of 8-50 base pairs having dyad symmetry.

22. The method of claim 21, wherein said heterologous DNA segment is the operator of the E. coli lac operon.

23. The method of claim 21, wherein said heterologous DNA segment comprises the following palindromic sequence:

ATTGTGAGCGCTCACAAT
TAACACTCGCGAGTGTTA

24. The method of claim 20, wherein one to ten copies of said heterologous DNA segment are present in said untranslated region.

25. The method of claim 24, wherein the number of said copies is one, two, or three.

26. The method of claim 20, wherein said transgene would, in the absence of said heterologous DNA segment, be expressed in said animal at an unattenuated level that would render normal sexual propagation of said animal non-feasible.

27. The method of claim 26, wherein said unattenuated level of expression of said transgene would prevent said animal from reaching sexual maturity.

28. The method of claim 27, wherein said unattenuated level of expression of said transgene would be fatal to said animal.

29. A method of making a operator$^+$/ repressor$^+$ transgenic non-human vertebrate animal having cells containing a first transgene, the level of expression of which is attenuated, said method comprising

(1) providing a transgenic non-human vertebrate recipient animal having cells containing a second transgene encoding a repressor protein, which second transgene was introduced into said recipient animal, or an ancestor of said animal, at an embryonic stage; and

(2) introducing said first transgene into a descendant of said recipient animal, at an embryonic

stage, said first transgene comprising an operator sequence to which said repressor protein is capable of binding, thereby reducing the level of expression of said first transgene.

30. The method of claim 29, wherein said binding, and thus said attenuation, may be reversed by introducing into said operator$^+$/repressor$^+$ animal an inactivator of said repressor protein.

31. The method of claim 29, wherein said attenuation may be reversed by mating said operator$^+$/repressor$^+$ animal with a second animal which is not transgenic for said repressor protein, and obtaining offspring of said mating which are operator$^+$/repressor$^-$.

32. The method of claim 29, wherein said repressor protein comprises the E. coli lac repressor protein.

33. The method of claim 31, wherein said operator sequence comprises either the operator of the E. coli lac operon or the following palindromic sequence:

ATTGTGAGCGCTCACAAT
TAACACTCGCGAGTGTTA

34. The method of claim 31, wherein said inhibitor is IPTG.

35. The method of claim 29, wherein said first transgene encodes IL-4 or human growth hormone.

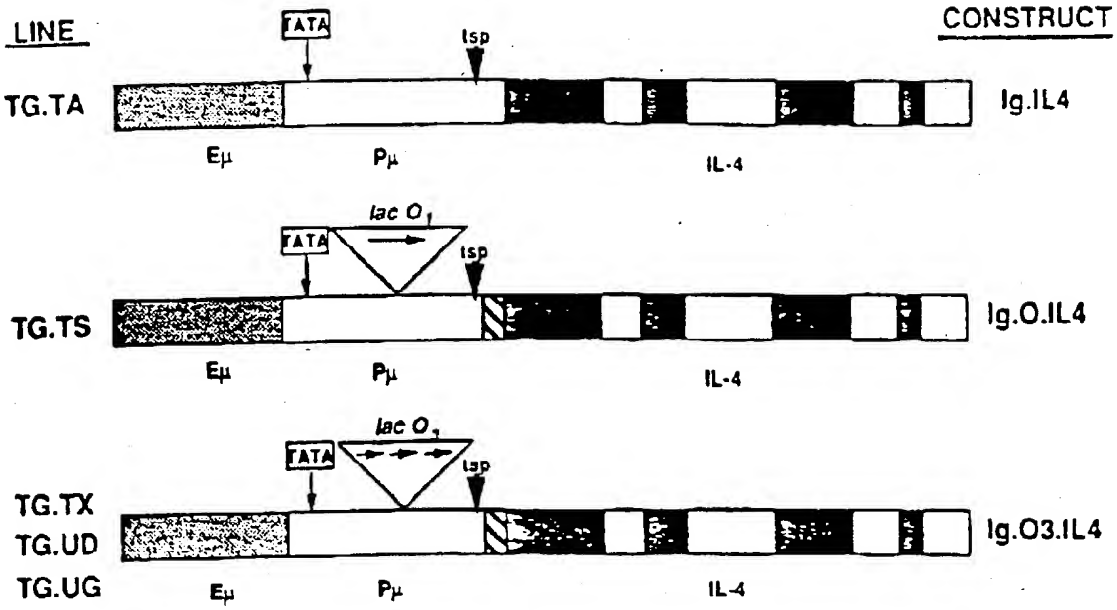
36. The method of claim 29, wherein said introduction of said first transgene into said descendant of said recipient animal is accomplished by sexually crossing a transgenic non-human vertebrate animal, the germline cells of which contain said first transgene, with said recipient animal.

37. The method of claim 29, wherein said introduction of said first transgene into said descendant of said recipient animal is accomplished by asexually inserting said first transgene into the genome of said descendant of said recipient animal, at an embryonic stage.

38. A method of testing an anti-allergy treatment, said method comprising providing the transgenic animal of claim 7, exposing said transgenic animal to said treatment, and determining the effect of said treatment on the allergic response of said transgenic animal.

39. A transgenic non-human vertebrate animal made by the method of claim 20 or claim 29.

FIG. 1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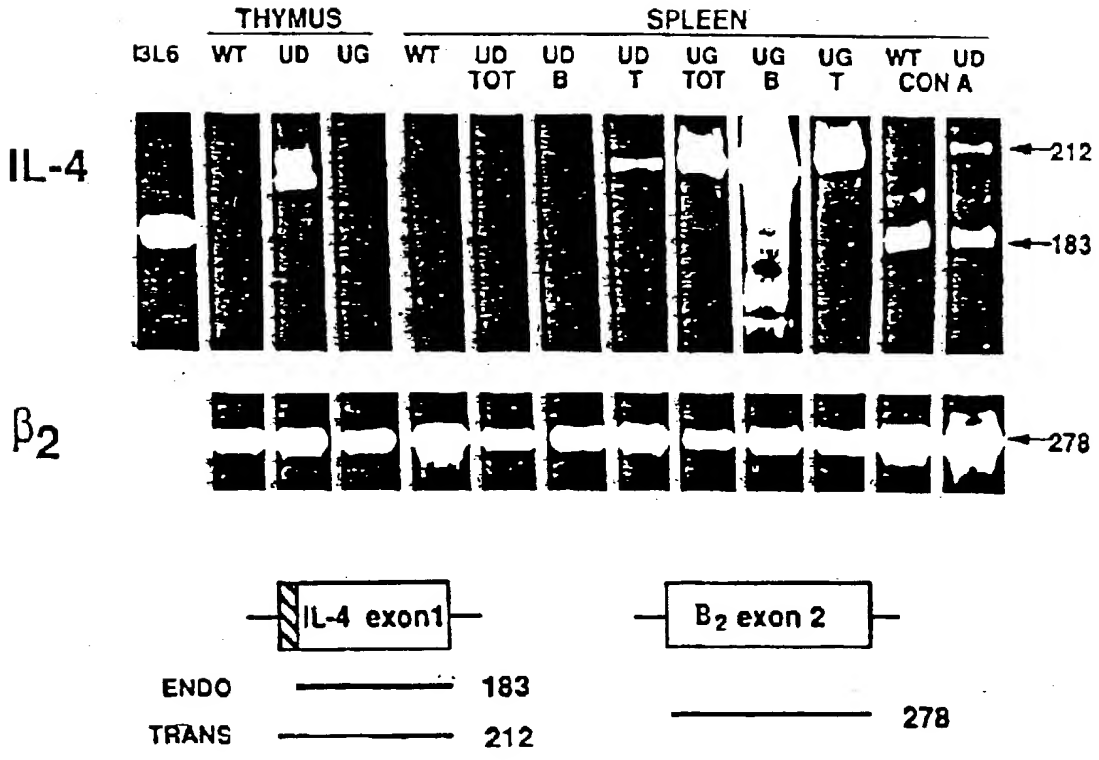
lsp
TG.TA
Ig.IL4
Eμ
Pμ
IL-4
lac O1
TATA
lsp
TG.TS
Ig.O.IL4
Eμ
Pμ
IL-4
lac O3
TATA
lsp
TG.TX
TG.UD
TG.UG
Ig.O3.IL4
Eμ
Pμ
IL-4

Figure 2

ATTGTGAGCGCTCACAAT
TAACACTCGCGAGTGTTA

FIG. 3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SPLEEN
B6
WT
UD
UG
WT
UD TOT
UD B
UD T
UG TOT
UG B
UG T
WT CON
UD A
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183
β2
278
IL-4 exon1
B2 exon 2
ENDO
183
TRANS
212
278

FIG. 4

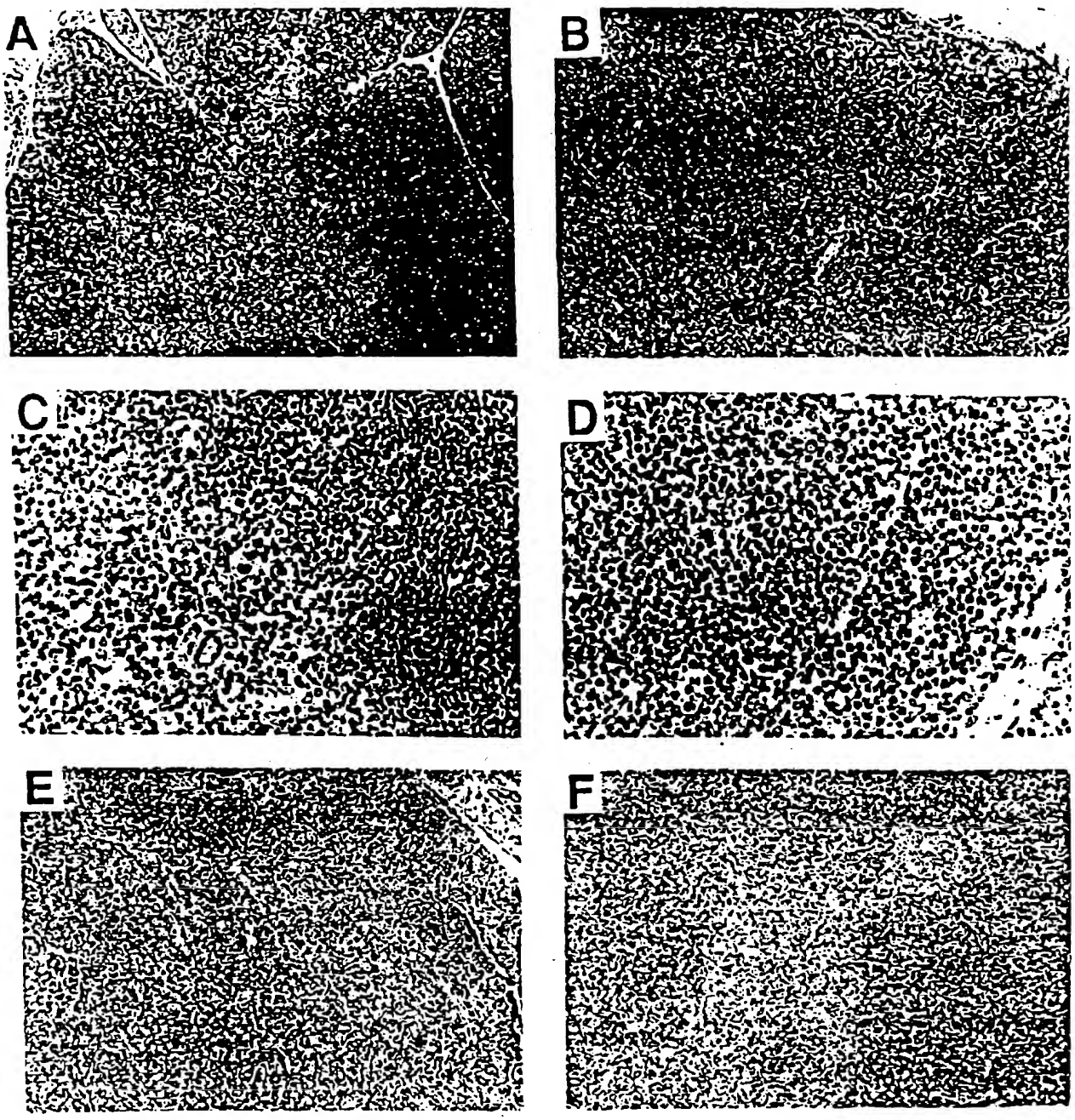
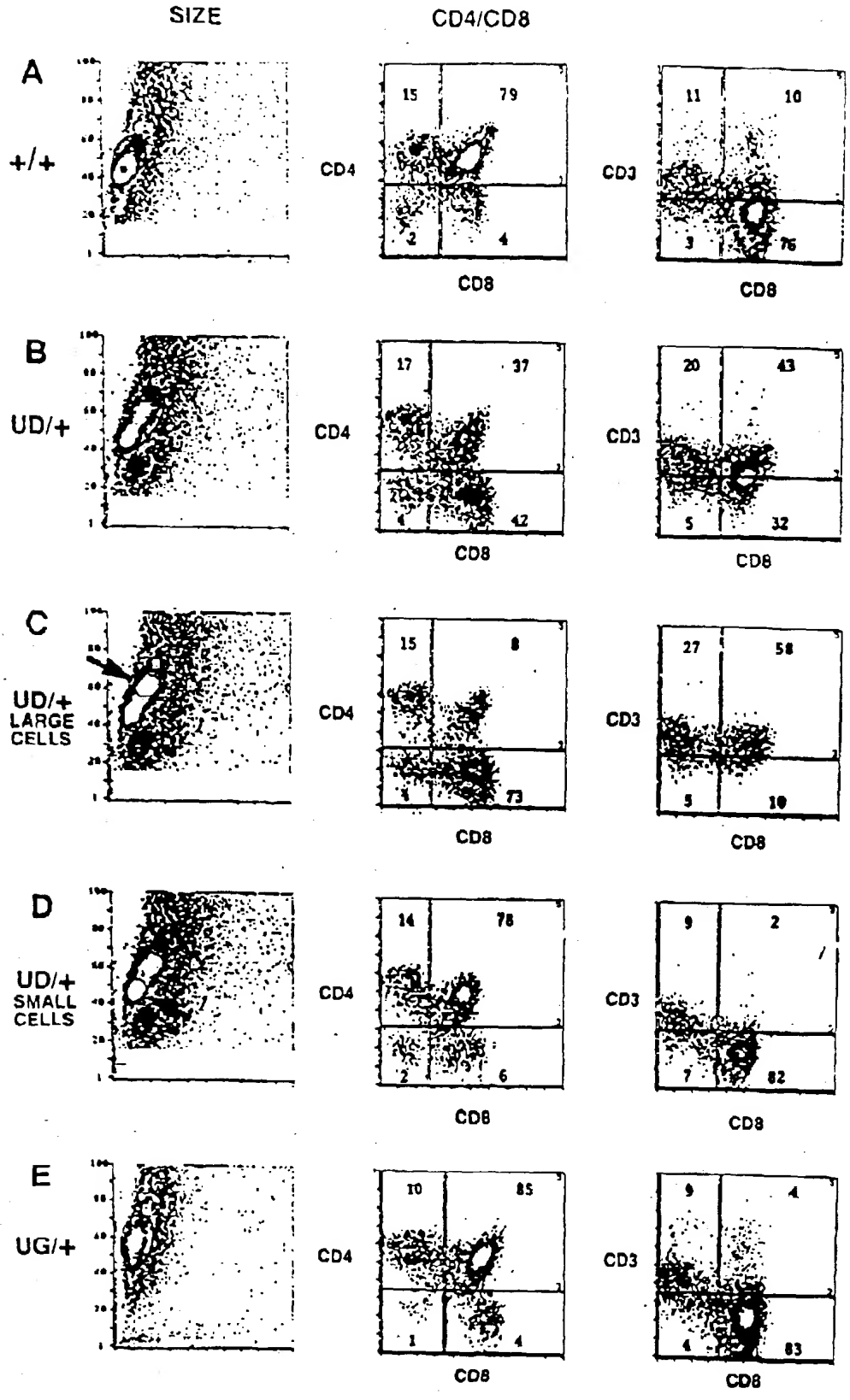


FIG. 5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CD8
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2
4
CD3
CD8
11
10
3
76
B
UD/+
CD4
CD8
17
37
4
42
CD3
CD8
20
43
5
32
C
UD/+
LARGE
CELLS
CD4
CD8
15
8
73
CD3
CD8
27
58
5
10
D
UD/+
SMALL
CELLS
CD4
CD8
14
78
2
6
CD3
CD8
9
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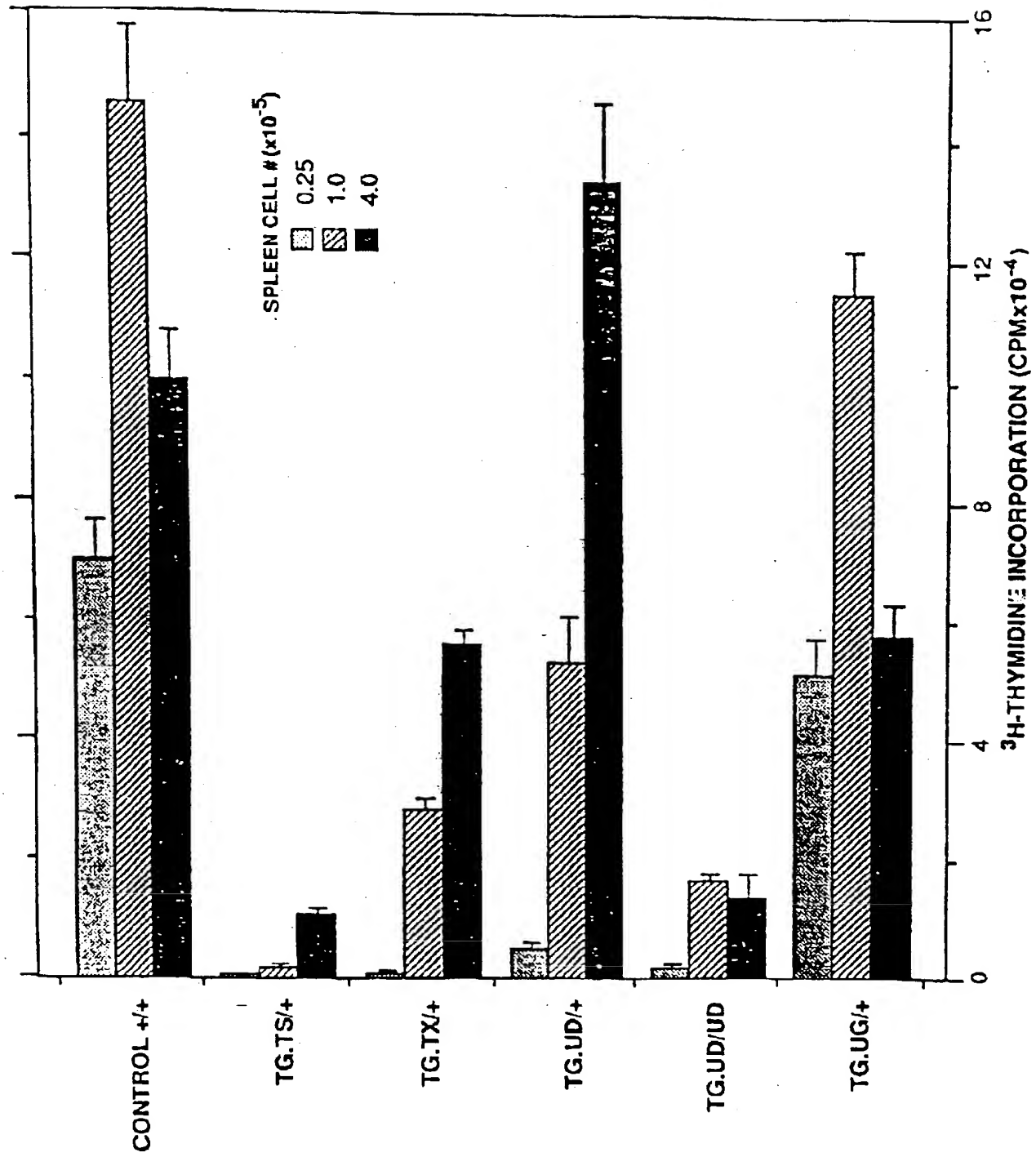
CD8
9
4
4
83

FIG. 6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0.25
1.0
4.0
^{3}H-THYMIDINE INCORPORATION (CPMx10^{-4})
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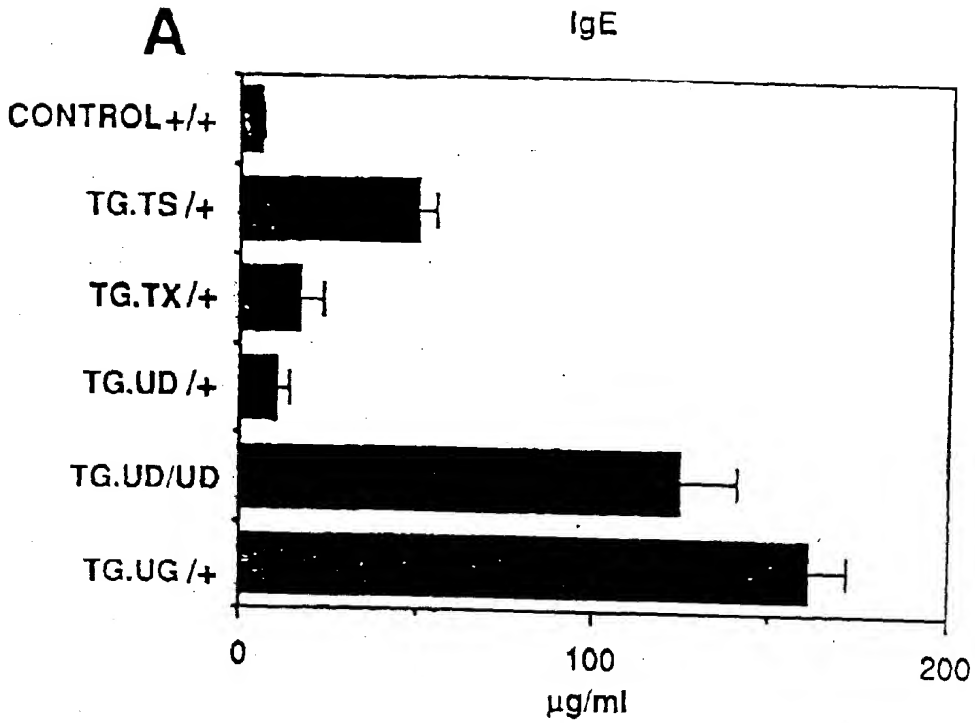
12
16
CONTROL +/+
TG.TS/+
TG.TX/+
TG.UD/+
TG.UD/UD
TG.UG/+

FIG. 7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IgE
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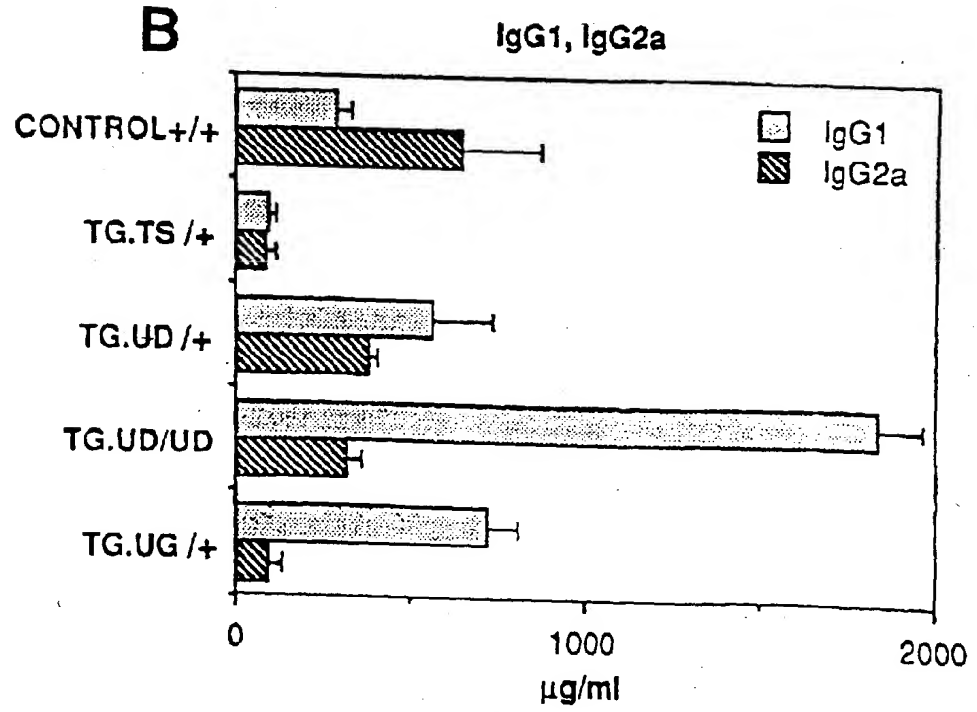
TG.TS /+
TG.TX/+
TG.UD /+
TG.UD/UD
TG.UG /+
0
100
200
µg/ml


B
IgG1, IgG2a
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IgG2a
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TG.TS /+
TG.UD /+
TG.UD/UD
TG.UG /+
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2000
µg/ml

A

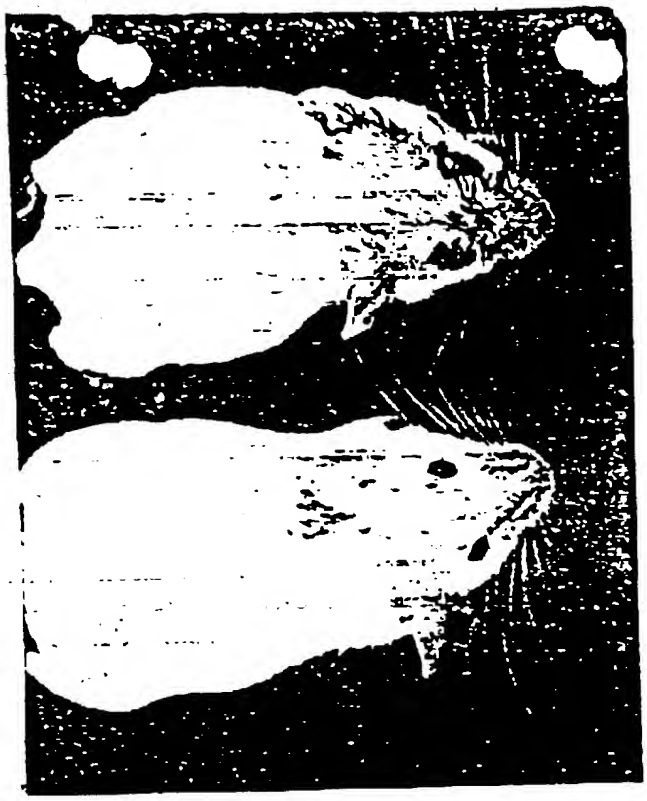
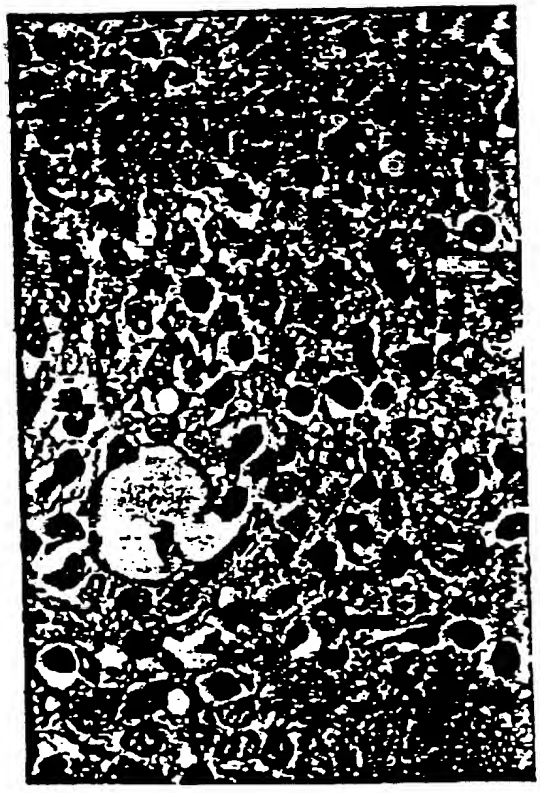


FIG. 8

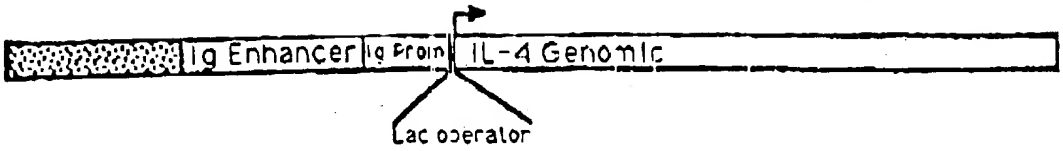
B



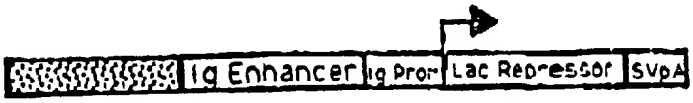
C

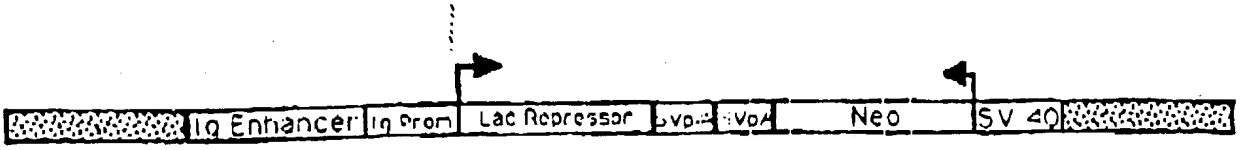
Figure 9

Ig-operator- Il-4



Ig-lac repressor





INTERNATIONAL SEARCH REPORT

International Application No PCT/US9101279

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00

U.S. Cl.: 800/2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	800/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

SEE ATTACHMENT

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,736,866 (Leder et al.) 12 April 1988, see entire document.	1-8,19,38
P, X / Y	Cell, vol. 62, issued 10 August 1990, Tepper et al., "IL-4 Induces Allergic-Like Inflammatory Disease And Alters T Cell Development In Transgenic Mice", pages 457-467, See entire document.	1-8,19 / 1-8,19,38
Y	Cell, vol. 57, issued 05 May 1989, Tepper et al., "Murine Interleukin-4 Displays Potent Anti-Tumor Activity In Vivo", pages 503-512, See entire document.	1-8,19,38
Y	Proc. Natl. Acad. Sci. USA, vol. 86, issued October 1989, Suemat'su et al., "IgG1 Plasmacytosis In Interleukin 6 Transgenic Mice", pages 7547-7551, see entire document.	1-8,19,38
Y	J. Immunol., vol. 140, issued 15 June 1988, Del Prete et al., "IL-4 Is An Essential Factor For The IgE Synthesis Induced In Vitro, By Human T Cell Clones And Their Supernatants", pages 4193-4198. see entire document.	1-8,19,38

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 March 1991	24 APR 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Christopher Low

Form PCT/ISA/210 (second sheet) (rev. 11.87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Proc. Natl. Acad. Sci. USA, vol. 85, issued March 1988, Shimizu et al., "Human And Rat Mast Cell High-Affinity Immunoglobulin E Receptors: Characterization Of Putative a-Chain Gene Products", pages 1907-1914., see entire document.	1-8,19,38

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

SEE ATTACHMENT

1 ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2 ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

I-8, 19, 38

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Serial Number PCT/US91/01279 (01/101.279)
Art Unit 184
Attachment to Form PCT/ISA/210 (second sheet)

II. FIELDS SEARCHED
Documentation searched other than minimum documentation to the extent that such documents are included in the Fields Searched.

U.S. Patent and Trademark Office Automated Patent System
DIALOG - BIOSIS PREVIEWS, CHINESE PATENT ABSTRACTS, CLAIMS(TM)/U.S. PATENTS, INPADOC/FAMILY AND LEGAL STATUS, WORLD PATENTS INDEX

Serial Number PCT/US91/01279 (01/101,279)
Art Unit 184
Attachment to Form PCT/ISA/210 (supplemental sheet)
OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

I. Claims 1-8, 19, and 38, drawn to a non-human transgenic animal having a heterologous IL-4 DNA and a method of testing anti-allergy treatment are for example, classified in Class 800, subclass 2, Class 435, subclass 172.3, and Class 424, subclass 9.

II. Claims 9-13, 19, 20-28, and 39 drawn to a non-human transgenic animal having a six base pair untranslated heterologous DNA selected from an operator sequence, a eukaryotic transcription binding site, a palindromic sequence, or a sequence with dyad symmetry and a method of making a non-human transgenic animal where expression of the heterologous DNA is attenuated and where 6-100 base pairs of DNA encode an attenuation function and is selected from an operator sequence, a eukaryotic transcription binding site, a palindromic sequence, or a sequence with dyad symmetry are, for example, classified in Class 800, subclass 2, and Class 435, subclasses 91, 172.1., 172.3, 320.1, and Class 536, subclass 27.

III. Claims 14-18, 19, 29-37, and 39 drawn to a non-human transgenic animal having a heterologous repressor protein and a method of making an operator$^+$/repressor$^-$ non-human transgenic animal where the level of the first heterologous DNA is attenuated and the second heterologous DNA encodes a repressor protein that effects lower expression of the first heterologous DNA and are both in one animal where an operator$^+$/repressor$^-$ animal is obtained by mating with a non-human transgenic animal that is repressor$^-$ are, for example, classified in Class 800, subclass 2 and Class 435, subclasses 172.1, 172.3, 320.1, and Cass 536, subclass 27.